

PHYSIOLOGY AND MANAGEMENT

Effects of Florfenicol, Chloramphenicol, and Thiamphenicol on Phagocytosis, Chemiluminescence, and Morphology of Bovine Polymorphonuclear Neutrophil Leukocytes¹

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ABSTRACT

Florfenicol, chloramphenicol, and thiamphenicol were tested in vitro to determine their potential toxic effects on bovine neutrophils. Antibiotics were tested at 4000, 2000, and 10 µg/ml of incubation mixture. Percentage phagocytosis was determined by incubations with neutrophils isolated from milk of five cows and ³²P-labeled *Staphylococcus aureus* and 5% skim milk. The effect of 4000 µg of each antibiotic on chemiluminescence was determined on neutrophils isolated from mammary secretions of three nulliparous heifers. Morphological evaluation by transmission and scanning electron microscopy was performed on neutrophils isolated from two heifers at antibiotic concentrations of 4000 and 10 µg/ml. Chloramphenicol depressed phagocytosis at the high and medium doses and blocked chemiluminescence activity at the high dose. No effects were observed for florfenicol and thiamphenicol. Transmission electron microscopic examination showed that at the high concentration of drugs, 99, 99, 97, and 76% of the neutrophils treated with florfenicol, chloramphenicol, thiamphenicol, and dimethyl sulfoxide were abnormal. Examination by scanning elec-

tron microscopy showed that the percentage of neutrophils without pseudopodia averaged 67, 94, 32, and 16%, respectively. Results indicated that neither florfenicol nor thiamphenicol altered neutrophil function, but they did alter neutrophil morphology, although to a lesser extent than did chloramphenicol.

(Key words: antibiotics, neutrophils, phagocytosis)

INTRODUCTION

Previous studies have shown that chloramphenicol had detrimental effects on bovine polymorphonuclear neutrophil phagocytosis (11, 25) and morphology (11). Because the drug causes aplastic anemia in humans, its use in the United States in food-producing animals is prohibited. However, chloramphenicol is approved for use in several European countries, and evidence indicates that veterinarians and others in the United States are using the drug in food-producing animals (17). Two analogs of chloramphenicol, florfenicol and thiamphenicol, have been synthesized. The major difference between the analogs and chloramphenicol is that the *p*-nitro group is replaced by a methyl sulfonyl group (Figure 1). The *p*-nitro group has been implicated with irreversible inhibition of growth of bone marrow precursor cells (15). Widespread use of thiamphenicol in Europe has not been associated with aplastic anemia (17). Both analogs possess an antimicrobial spectrum similar to that of chloramphenicol (10, 20). However, florfenicol has been reported (20) to possess superior in vitro bactericidal activity compared with chloramphenicol and thiamphenicol. This has been at-

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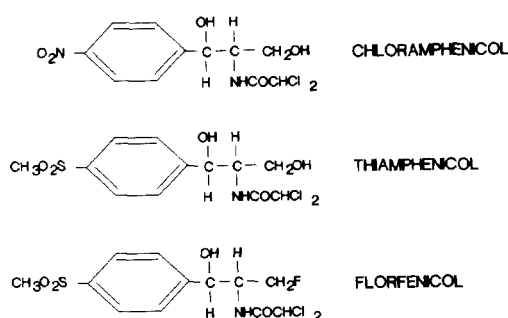


Figure 1. Chemical structure of chloramphenicol, thiamphenicol, and florfenicol.

tributed to the substitution of a fluorine atom for the hydroxyl group on the 3' carbon. The efficacy of intramammary florfenicol for the treatment of mastitis in lactating cows is currently under investigation (G. Ziv, unpublished observations).

The purpose of this study was to determine in vitro effects of chloramphenicol, florfenicol, and thiamphenicol on phagocytosis, chemiluminescence, and morphology of bovine neutrophils.

MATERIALS AND METHODS

Cattle

Five lactating cows and three nulliparous heifers were used as sources of neutrophils. On the basis of results of bacteriologic culture of mammary secretions on blood esculin agar, these cattle were free of intramammary pathogens.

Each of two separate trials consisted of phagocytosis measurements on mammary gland neutrophils from three and two lactating cows, and three separate trials consisted of chemiluminescence (CL) and morphologic measurements on mammary neutrophils collected alternatively from one of the three heifers.

Drugs

Chloramphenicol (Sigma Chemical Co., St. Louis, MO), thiamphenicol (Shering-Plough

Corp., Union, NJ), and florfenicol (Shering-Plough Corp.) were dissolved in dimethyl sulfoxide (DMSO) (Sigma Chemical Co.) and diluted to desired concentrations in either balanced salt solution or .01 M phosphate buffered saline (PBS).

Neutrophils

Mammary gland neutrophils were isolated from lactating cows 14 h after intramammary inoculation of 50 µg of *Escherichia coli* endotoxin (*E. coli* 026:B6, Boivin TCA procedure, endotoxin code 3920, Difco Laboratories, Detroit, MI) in 50 ml of sterile PBS. To induce milk ejection, 7 IU of oxytocin (Anpro Pharmaceutical, Arcadia, CA) were administered into the tail vein, and 250 ml of milk were collected (by hand milking) into a polypropylene flask. Samples and reagents were kept at 5°C during the isolation procedure. Milk was filtered through silk cloth (NSG Precision Cells, Inc., Hicksville, NY), poured into siliconized bottles (Cores, Du Pont Co., Newtown, CT), and centrifuged at 1000 × *g* for 15 min. The cream layer was removed, the skim milk was discarded, and the cell pellet was gently suspended in 80 ml of PBS. After centrifugation at 200 × *g*, neutrophils were washed again in PBS. The suspension was centrifuged again, and the sedimented cells were suspended in PBS and then adjusted to 25 × 10⁶/ml. On the average, 97% of the cells recovered were neutrophils with 95% viability as determined by Trypan blue exclusion.

Neutrophils from the mammary gland of nulliparous heifers were isolated after intramammary inoculation of 5 µg of *E. coli* endotoxin in 5 ml of PBS. At 14 h after endotoxin administration, 20 ml of PBS were inoculated into the endotoxin-treated quarters, and mammary secretions were collected into a polypropylene flask. The neutrophils were isolated and their concentration was adjusted, similarly as for those from lactating cows. Typically, 94% of the isolated cells were neutrophils, of which 96% were viable.

Phagocytosis

Percentage of phagocytosis was measured after a 60-min incubation as described (7). Drugs were tested at in vitro concentrations of

4000, 2000, and 10 $\mu\text{g/ml}$ of incubation medium. The final concentrations of DMSO in the incubation medium were 6.25, 3.12, and .02%. The incubation mixture included .5 ml of drug or corresponding dilution of DMSO, .5 ml of 10% skimmed milk in PBS, .5 ml of the neutrophil suspension, and .5 ml of ^{32}P -labeled *Staphylococcus aureus* (2×10^6). All determinations were performed in triplicate.

Chemiluminescence

Balanced salt solution was prepared as previously described (8), except that phenol red was not added. The solutions and methodology used were as previously described (6). Luminol-enhanced CL was measured in a liquid scintillation counter (LS 100C, Beckman Instruments, Inc., Silver Spring, MD) at 21°C, using the tritium channel and the in-coincidence mode. Isolated mammary gland neutrophils ($5 \times 10^6/.2$ ml) from a nulliparous heifer were added to 7-ml scintillation vials containing luminol (1.8 ml), 1 ml of zymosan (5×10^8 particles/ml), and 1 ml of drug or DMSO in balanced salt solution. Final concentrations of drug and DMSO in the incubation mixture were 4000 $\mu\text{g/ml}$ and 6.25%. Vials were counted for 1 min at 16-min intervals for a maximum of 128 min. All determinations were performed in duplicate.

Morphologic Study

Neutrophils (12.5×10^6) collected from mammary secretions of two nulliparous heifers were incubated for 2 h at 37°C at the high and low concentrations of antibiotics and DMSO. Cells were then fixed in 2.5% glutaraldehyde in .1 M cacodylate buffer (pH 7) at room temperature, fixed as described (21), and dehydrated in a graded series of ethanol. For light microscopy, neutrophils were embedded in Spurr's medium (18) and sectioned. For transmission electron microscopy, ultrathin sections (60 to 90 nm) were stained with uranyl acetate and lead citrate and examined, using an electron microscope operating at 60 kV. A morphologic analysis was performed on leukocytes incubated with each drug in culture medium, and values were compared with those of DMSO controls. Cells were classified as previously described (11): 1) normal, cells with a multilobed nucleus

and numerous pseudopodia; 2) altered, characterized by slight rounding of the cell and nuclear lobes with partial loss of pseudopodia; 3) swollen, gross rounding of nuclear lobes and complete rounding of the cell; and 4) lysed, characterized by nuclear swelling, extrusion of cell contents, and partial loss of the plasma membrane. The number of cells with cytoplasmic vacuoles and with cytoplasmic blebs protruding from the cell surface were also recorded. Two hundred cells per treatment were characterized. For scanning electron microscopy, fixed cells were processed (9) and 200 cells were examined using a scanning electron microscope operating at 20 kV. The number of cells with no pseudopodia (rounded) was recorded.

Statistical Analyses

One-way analysis of variance procedures were used to make comparisons of the means and percentage change in phagocytosis from DMSO control. Groups were drug and concentration combinations. Chemiluminescence data were analyzed by general linear models procedures of SAS (SAS Institute, Cary, NC) with a model including drug, incubation time, and interaction of drug \times time. The tests of significance were based on log (base₁₀) of counts per minute (mean of two counts). For morphologic results, comparisons were made separately for each concentration of each antibiotic versus DMSO by each morphologic category, by two-way factorial chi-square analysis.

RESULTS

Phagocytosis

A significant ($P < .05$) inhibitory effect on phagocytosis was observed for chloramphenicol at the high and intermediate drug concentrations (Table 1). Florfenicol and thiamphenicol had no effect ($P > .05$) at any of the concentrations tested.

Chemiluminescence

Activity of neutrophils was depressed ($P < .05$) by chloramphenicol between 16 and 112 min compared with DMSO (Figure 2). Although CL activity compared with DMSO

TABLE 1. Effect of chloramphenicol analogues on phagocytosis.¹

Drug	Concentration, $\mu\text{g/ml}$		
	4000	2000	10
	————— (%) —————		
Chloramphenicol	-16.6 ^a	-18.6 ^a	4.3
Florphenicol	-7.8	-3.0	3.3
Thiamphenicol	-5.2	.8	3.7

^aDifferent ($P < .05$) from 6.25, 3.125, and .02% dimethyl sulfoxide control values of 31.8, 36.4, and 47.6%.

¹Data expressed as percentage of change relative to DMSO controls.

was reduced by florfenicol and thiamphenicol, the differences were not significant ($P > .05$). Maximum CL activity occurred at 48 min of incubation.

Morphology Study

Transmission electron microscopic examination of cells incubated in drugs and DMSO indicated that most cells had altered morphology (Table 2). At the high concentration of drug, fewer ($P < .01$) normal neutrophils were observed than with DMSO. More normal cells were observed at the lowest concentration of drug. Interestingly, the percentage of normal cells for florfenicol (54%) and DMSO (58%) were very similar, although not different ($P > .05$) from chloramphenicol (37%) and thiamphenicol (33%). Cytoplasmic vacuoles were commonly observed in neutrophils for all concentrations of drugs and DMSO (Table 3, Figures 3, 4, and 5). Fewer ($P < .05$) neutrophils

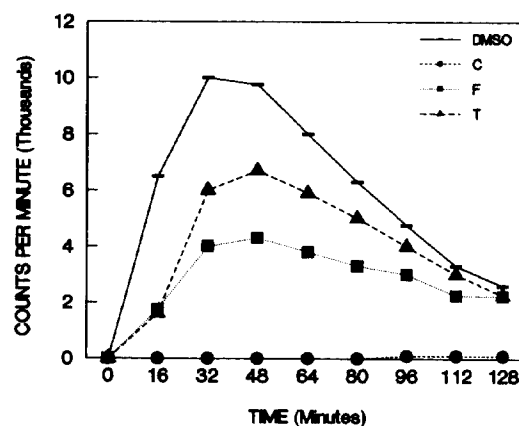


Figure 2. Comparison of chemiluminescence activity of bovine neutrophils after exposure to 4000 $\mu\text{g/ml}$ of chloramphenicol (C), florfenicol (F), thiamphenicol (T), and dimethyl sulfoxide (DMSO).

were observed with cytoplasmic vacuoles when incubated in the lowest concentration of florfenicol (Table 3). However, more ($P < .01$) neutrophils were observed with cytoplasmic vacuoles at the high concentration of florfenicol and chloramphenicol.

A commonly observed abnormality was the blebbing of cytoplasm from the surface of neutrophils incubated in the high concentration of florfenicol (Figure 5C). Significantly more neutrophils (26%) incubated in the high dose of florfenicol had blebbing than in the DMSO control (Table 3). Although a high percentage of neutrophils incubated in the low (20%) and high (18%) doses of thiamphenicol also had

TABLE 2. Effect of antibiotics on neutrophil morphology as determined by transmission electron microscopy.

Drug	Concentration, $\mu\text{g/ml}$							
	Normal		Altered		Swollen		Lysed	
	10	4000	10	4000	10	4000	10	4000
	————— (%) —————							
Chloramphenicol	37	1 ^a	52	83	8	9	3	7
Florphenicol	54	1 ^a	34	69	3	19	9	11
Thiamphenicol	33	3 ^a	58	72	7	16	2	9
DMSO ¹	(58)	(24)	(33)	(63)	(2)	(6)	(7)	(7)

^aValues within each drug concentration are different from DMSO control ($P < .01$).

¹Concentrations of dimethyl sulfoxide were .02 and 6.25%.

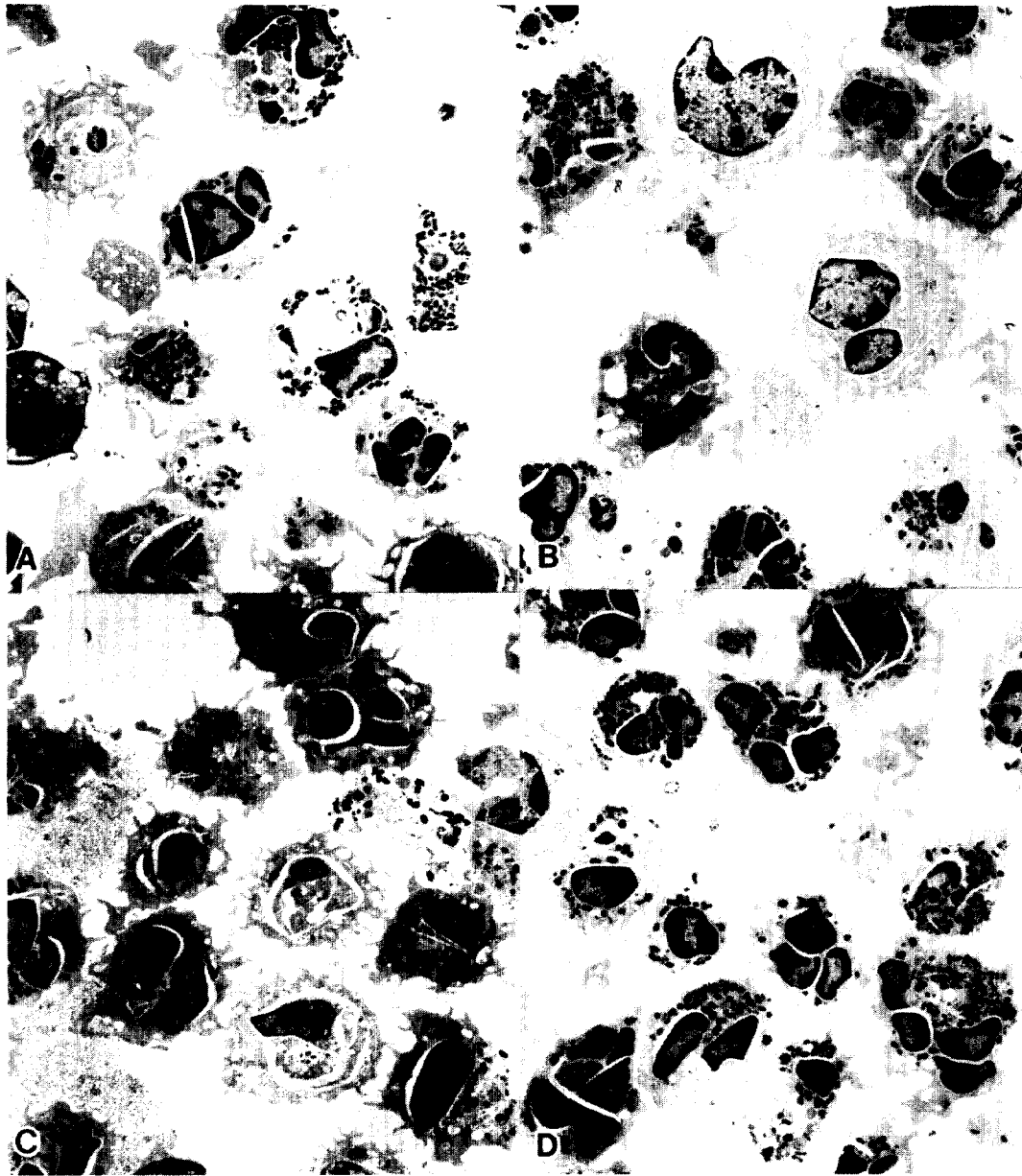


Figure 3. Transmission electron micrographs of neutrophils incubated in media containing A) .02% dimethyl sulfoxide (DMSO), B) 10 μ g/ml of chloramphenicol, C) 10 μ g/ml of florfenicol, and D) 10 μ g/ml of thiamphenicol. Neutrophils incubated in chloramphenicol (B), florfenicol (C), and thiamphenicol (D) appeared morphologically similar to those incubated in DMSO (A). $\times 3900$.

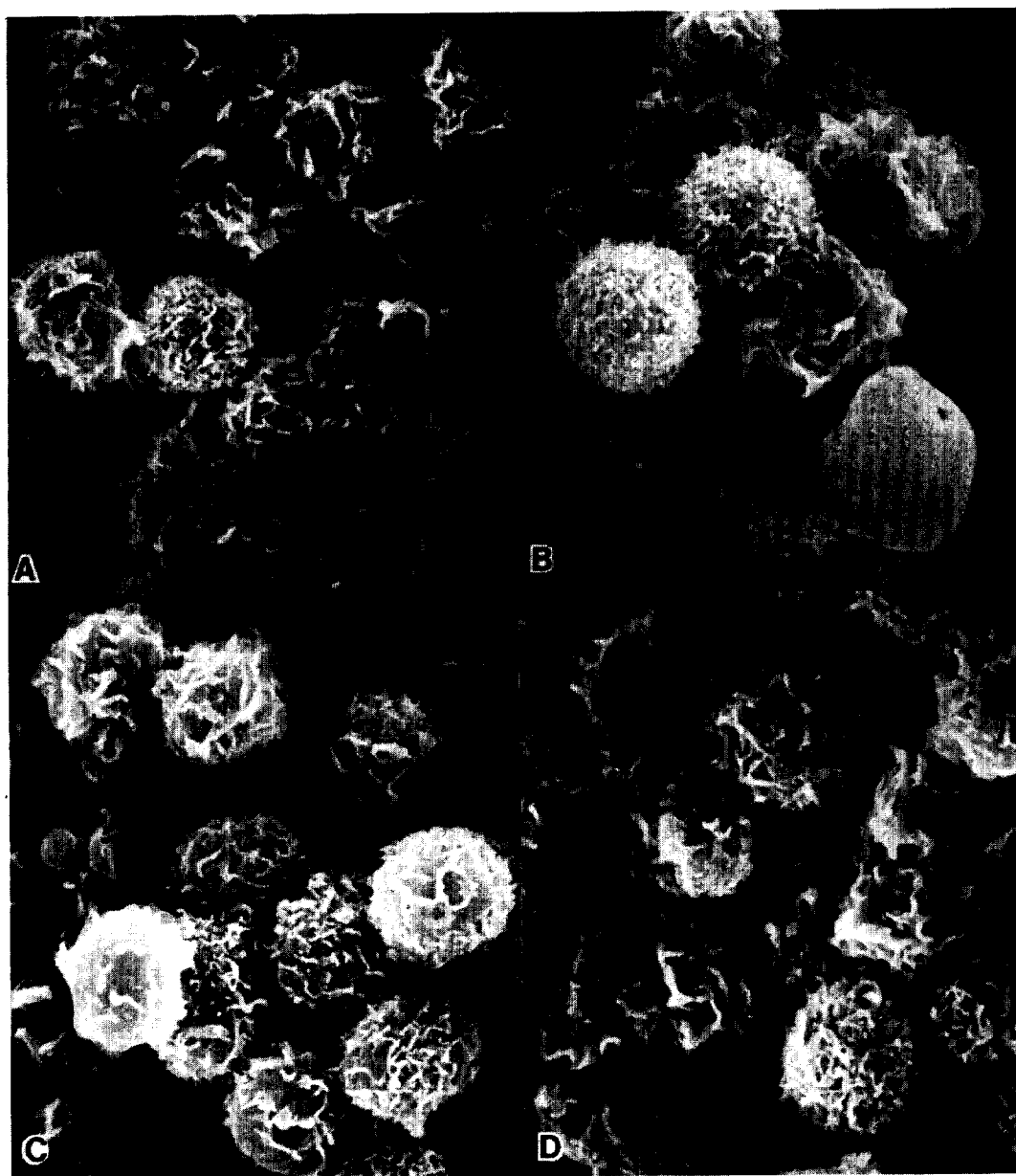


Figure 4. Scanning electron micrographs of neutrophils incubated in media containing A) .02% dimethyl sulfoxide (DMSO), B) 10 µg/ml of chloramphenicol, C) 10 µg/ml of florfenicol, and D) 10 µg/ml of thiamphenicol. Pseudopodia were not as distinct on neutrophils incubated in chloramphenicol (B). Rounded cells can be seen at the bottom for this cluster of cells. The cell surfaces for neutrophils incubated in florfenicol (C) and thiamphenicol (D) were similar to those for neutrophils incubated in DMSO (A). $\times 4000$.

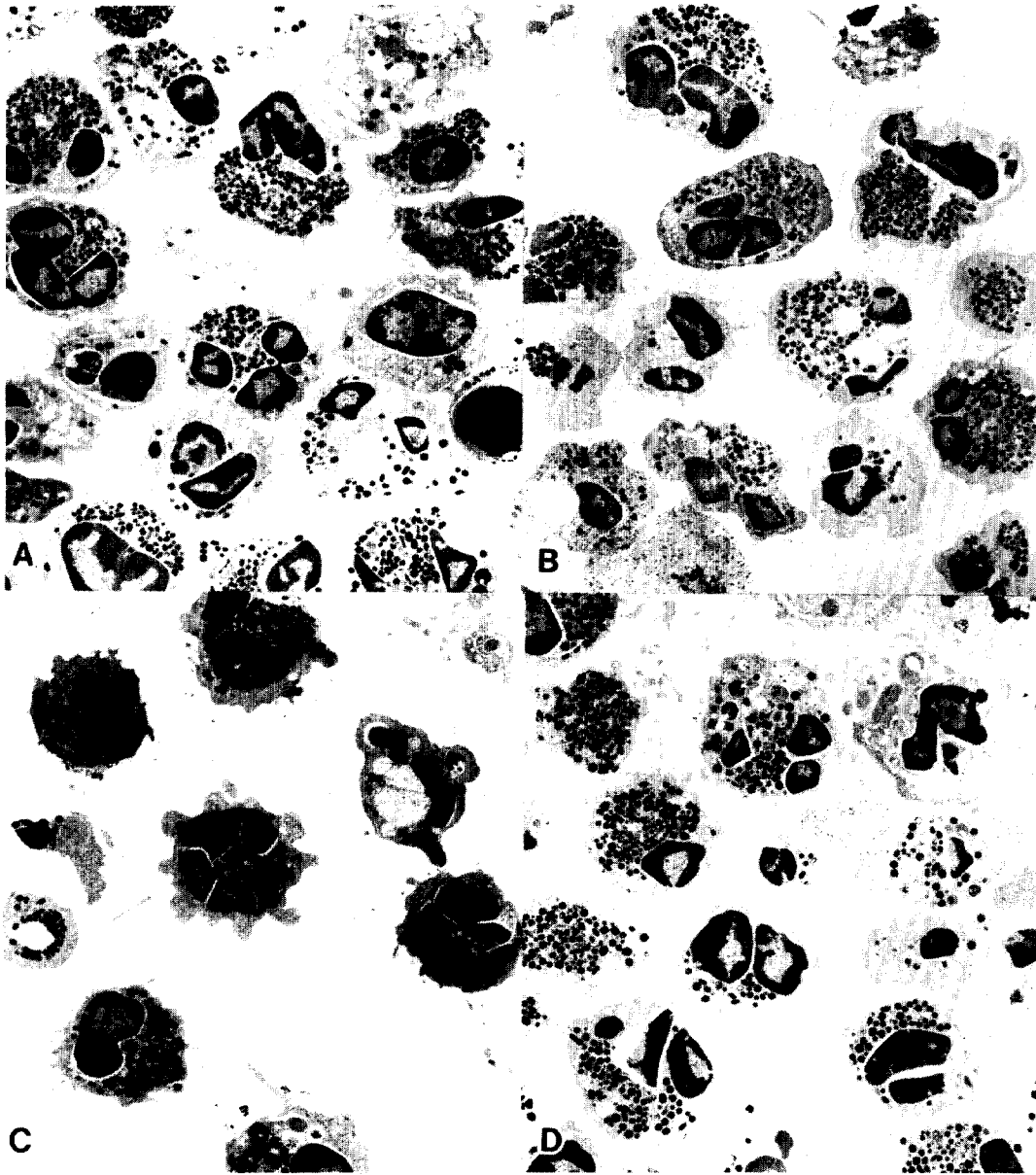


Figure 5. Transmission electron micrographs of neutrophils incubated in media containing A) 6.25% dimethyl sulfoxide (DMSO), B) 4000 µg/ml of chloramphenicol, C) 4000 µg/ml of florfenicol, and D) 4000 µg/ml of thiamphenicol. Severe rounding of the cells is apparent for neutrophils incubated in chloramphenicol (B). Blebbing of the cytoplasm is clearly visible protruding from surfaces of neutrophils incubated in florfenicol (C). Compared with neutrophils incubated in DMSO (A), a condensing of the granular content away from the periphery of the cells is apparent in neutrophils incubated in chloramphenicol (B) and florfenicol (C), and to a lesser extent in neutrophils incubated in thiamphenicol (D). $\times 3900$.

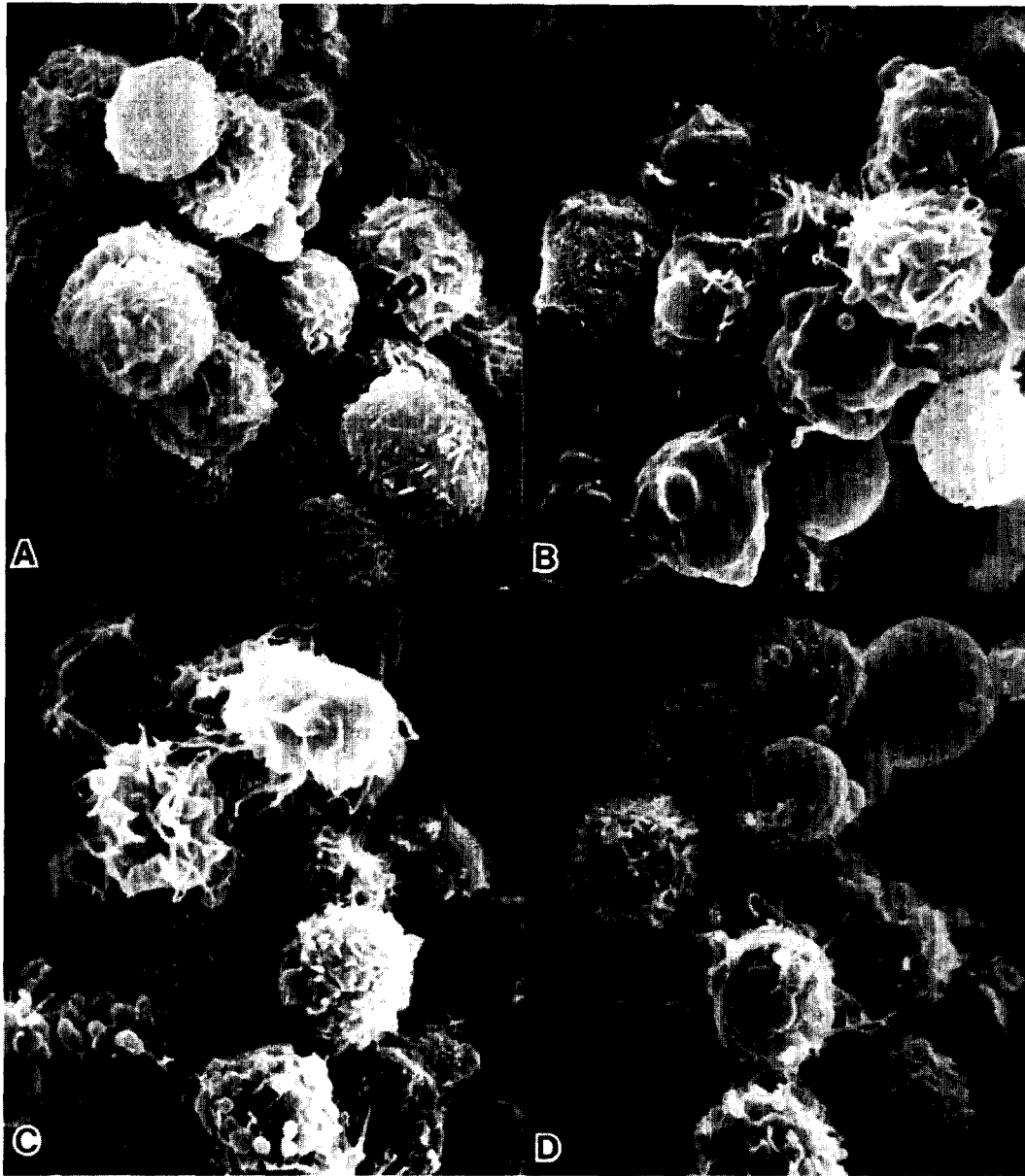


Figure 6. Scanning electron micrographs of neutrophils incubated in media containing A) 6.25% dimethyl sulfoxide, B) 4000 µg/ml of chloramphenicol, C) 4000 µg/ml of florfenicol, and D) 4000 µg/ml of thiamphenicol. More rounded cells can be observed for neutrophils exposed to chloramphenicol (B). Blebs of cytoplasm are seen coming from the surface of neutrophils incubated in florfenicol (C) and thiamphenicol (D). $\times 3900$.

TABLE 3. Effect of antibiotics on neutrophil morphology as determined by transmission electron microscopy.

Drug	Concentration, $\mu\text{g/ml}$			
	Cytoplasmic vacuoles		Blebs	
	10	4000	10	4000
	(%)			
Chloramphenicol	44	54 ^a	5	9
Florphenicol	16 ^a	42 ^a	1	26 ^a
Thiamphenicol	36	26	20	18
DMSO in PBS ¹	(35)	(21)	(11)	(8)

^aValues within each drug concentration are different from DMSO control ($P < .01$).

¹Concentrations of dimethyl sulfoxide were .02 and 6.25% in phosphate-buffered saline.

cytoplasmic blebs, the percentages were not different from the DMSO controls.

Another commonly observed abnormality was the loss of pseudopodia from the surface of neutrophils incubated in the high concentration of chloramphenicol and florphenicol (Table 4, Figure 6). Counts made on 200 cells by scanning electron microscopy revealed that for cells incubated in 4000 $\mu\text{g/ml}$ of chloramphenicol and florfenicol 94 and 67% were rounded (Table 4), which was greater ($P < .01$) than what was observed for the DMSO control. No effect was observed for cells incubated in thiamphenicol. The distribution of pseudopodia on cells appeared normal for neutrophils incubated in low doses of drugs and DMSO (Table 4, Figure 4). However, pseudopodia for chloramphenicol-treated cells did not appear to extend out as far from the cell as did pseudopodia on cells incubated in other drugs or DMSO. Additional abnormalities included a greater rounding of the nuclear lobes and cytoplasmic granules in neutrophils incubated in the high concentrations of florfenicol (Figure 5C) compared with the other drugs at this concentration and with DMSO.

Transmission electron microscopic examination of neutrophils revealed a condensing of the cytoplasmic granules in neutrophils incubated in the high concentration of all three antibiotics (Figures 5B, C, and D). The movement of granules away from the periphery of the cells was readily discernible. This phenomenon was not observed in neutrophils incubated in the

TABLE 4. Effect of antibiotics on rounding of neutrophils as determined by scanning electron microscopy.

Drug	% Rounded neutrophils, concentration, $\mu\text{g/ml}$	
	10	4000
Chloramphenicol	5	94 ^a
Florphenicol	4	67 ^a
Thiamphenicol	4	32
DMSO ¹	(2)	(10)

^aSignificantly different from DMSO control ($P < .01$).

¹Concentrations of dimethyl sulfoxide were .02 and 6.25%.

low dose of antibiotics or in the low and high doses of DMSO.

DISCUSSION

The drug concentrations used in the present study represent what is currently found in milk during the first 12 h after intramammary treatment at recommended doses (24, 26). Intramammary chloramphenicol therapy at doses ranging between .5 and 5 g/quarter is used in many European and other countries (22) for the treatment of coliform mastitis. Thus, high concentrations of the drug are present in the milk soon after treatment. In the present study, 4000 and 2000 $\mu\text{g/ml}$ concentrations of chloramphenicol resulted in 16.6 and 18.6% reductions in phagocytosis. Previous studies (11, 25) using in vitro concentrations of 4000 $\mu\text{g/ml}$ reported much greater decreases in phagocytosis by mammary neutrophils, on the order of 47.9 to 38%. Variation among cows in sensitivity of neutrophils to chloramphenicol, or the source of the neutrophils, could explain the less drastic effects on phagocytosis observed in the present study. Variation exists among cows in the ability of neutrophils to phagocytose (2, 14). In vitro concentrations of 1000 $\mu\text{g/ml}$ or less are not inhibitory to phagocytosis (4). Importantly, parenterally administered chloramphenicol does not result in milk drug concentrations found to impede phagocytosis (23).

A significant observation was the lack of an inhibitory effect on phagocytosis by florfenicol and thiamphenicol at all concentrations tested. However, in a recent study (4), incubation of neutrophils in 5, 125, or 1000 $\mu\text{g/ml}$ concentra-

tions of florfenicol resulted in significant inhibition of phagocytosis by bovine neutrophils compared with 5% bovine serum in PBS. However, the florfenicol used in that study was in an organic solvent. The authors did not specify if appropriate dilutions were made of the solvent used to dissolve the florfenicol in the 5% serum control medium. Thus, the organic solvent used with the florfenicol and not florfenicol itself may have been responsible for the observed inhibitory effects on phagocytosis.

A striking finding was the complete blocking of CL activity of neutrophils by the 4000 µg/ml concentration of chloramphenicol. As a result of respiratory burst activation, neutrophils produce highly unstable oxygen metabolites that are involved in the killing of bacteria (3). Chemiluminescence, the accompanying light emission, can be measured spectrophotometrically and has been used to study oxygen-dependent microbicidal systems of neutrophils (1). Results from this investigation indicated that although neutrophils may still have been capable of microbial ingestion in medium containing 4000 µg/ml of chloramphenicol, oxidative killing mechanisms may be totally suppressed. Fortunately, although high concentrations of the drug are present in the milk soon after treatment, the drug is rapidly absorbed from the udder (27). Intramammary injection of 5 g of chloramphenicol into a lactating mammary quarter will result in a milk concentration of 115.9 µg/ml 2 h after injection (13). Thus, suppression of intracellular oxidative microbicidal systems may be of short duration. In a previous study (13), neutrophils exposed *in vivo* to chloramphenicol concentration of 414 µg/ml of milk showed no diminishment of CL activity. A favorable finding from the present study was the detection of CL activity in neutrophils after exposure to florfenicol and thiamphenicol. Depending on results from efficacy trials, results of these functional studies seem to indicate that both florfenicol and thiamphenicol should be considered as suitable therapeutic replacements for chloramphenicol.

Results from the morphologic study produced some unexpected findings. From the functional studies, we anticipated that florfenicol and thiamphenicol would be less damaging to neutrophils than would chloramphenicol. However, all three drugs altered the morphologic features of neutrophils. At the

highest concentration of drug the percentage of normal cells was only 1 to 3%. Almost all of the neutrophils (94%) exposed to the highest concentration of chloramphenicol had no pseudopodia. This finding suggests that disruption of the microtubule assembly occurred. Microtubules play a role in degranulation (5) and maintenance of pseudopodia (19). A classic example of the importance of microtubules in degranulation comes from results of studies on the disease, Chediak-Higashi Syndrome. Neutrophils from patients with this disease are unable to undergo normal degranulation (16). *In vitro* treatment of Chediak-Higashi Syndrome cells with Concanavalin A produces "capping" (12), which is construed as evidence of defective microtubule assembly. Furthermore, the significant inhibition of chloramphenicol on phagocytosis reported in this and other studies (11, 25) suggests inhibition of the assembly of actin monomers into filaments, a critical step for phagocytosis to occur (19).

CONCLUSIONS

Chloramphenicol suppressed phagocytosis and completely blocked respiratory burst activity of neutrophils, effects not observed in neutrophils exposed to florfenicol and thiamphenicol. All three drugs altered the morphology of neutrophils with effects being slightly greater in neutrophils treated with chloramphenicol. Because both florfenicol and thiamphenicol possess an antimicrobial spectrum similar to that of chloramphenicol, use of these two drugs as therapeutic agents appears appropriate.

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